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Preliminary crystallographic data of the three homologues of the thiol–disulfide oxidoreductase DsbA in *Neisseria meningitidis*

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Bacterial virulence depends on the correct folding of surface-exposed proteins, a process that is catalyzed by the thiol-disulfide oxidoreductase DsbA, which facilitates the synthesis of disulfide bonds in Gram-negative bacteria. Uniquely among bacteria, the *Neisseria meningitidis* genome possesses three genes encoding active DsbAs: DsbA1, DsbA2 and DsbA3. DsbA1 and DsbA2 have been characterized as lipoproteins involved in natural competence and in host-interactive biology, while the function of DsbA3 remains unknown. In an attempt to shed light on the reason for this multiplicity of *dsbA* genes, the three enzymes from *N. meningitidis* have been purified and crystallized in the presence of high concentrations of ammonium sulfate. The best crystals were obtained using DsbA1 and DsbA3; they belong to the orthorhombic and tetragonal systems and diffract to 1.5 and 2.7 Å resolution, respectively.

1. Introduction

Neisseria meningitidis is an invasive bacterial pathogen that causes life-threatening infection in children worldwide. The host–pathogen interactions and therefore the virulence depend on the correct folding of many surface-exposed proteins, a process that is catalyzed by the thiol-disulfide oxidoreductase DsbA, which performs the synthesis of disulfide bonds in the periplasm of Gram-negative bacteria (Bardwell *et al.*, 1991). This reaction is essential for the folding of virulence components, such as elements of the type IV secretion system, that require the formation of several disulfide bonds (Tinsley *et al.*, 2004) to be functionally active. While *Escherichia coli* and many other Gram-negative bacteria, including human pathogens such as *Shigella* and *Haemophilus*, have a single *dsbA* gene, *N. meningitidis* uniquely possesses three genes (named *nmb0278*, *nmb0294* and *nmb0407* in the sequenced genome of the serogroup B strain MC58) encoding three active DsbAs (DsbA1, DsbA2 and DsbA3), each of which shows different enzymatic properties (Sinha *et al.*, 2004; Tinsley *et al.*, 2004). DsbA1 and DsbA2 are membrane-associated lipoproteins, while DsbA3 is a soluble periplasmic protein like its well studied *E. coli* counterpart. Deletion of each of these genes has highlighted that the presence of at least one of the membrane-anchored enzymes is sufficient to restore full neisserial virulence (Tinsley *et al.*, 2004) or to maintain natural competence for DNA uptake at the wild-type level (Sinha *et al.*, 2008). In contrast, the role of the periplasmic enzyme seems to be unrelated to either of these phenotypes. The neisserial enzymes share 21% sequence identity with their *E. coli* homologue, the crystal structure of which is composed of one thioredoxin domain and one helical domain (Martin, Bardwell *et al.*, 1993). The three proteins are sufficiently similar to be defined unambiguously as DsbAs, but their primary sequences differ markedly, notably around the active-site domain (Fig. 1; Sinha *et al.*, 2004; Tinsley *et al.*, 2004). These differences, with their anticipated structural consequences, may underlie the distinct properties of each protein (Ondo-Mbele *et al.*, 2005). Strikingly, the presence of three *dsbA* genes is a feature of *N. meningitidis* alone among neisserial species. *N. gonorrhoeae*, *N. lactamica* and some

other commensals only possess homologues of *nmb0278* and *nmb0407*, while other commensals such as *N. flava* possess only one *dsbA*, a homologue of *nmb0294* (Sinha *et al.*, 2004). These differences may reflect the striking differences in host–pathogen interactive biology, which is plausibly related in part to the folding of the substrates of DsbA. In order to begin to understand the specific contributions of each of these enzymes to neisserial biology, we have initiated a structural study of these three enzymes; here, we present preliminary crystallographic data obtained on the three *N. meningitidis* DsbAs.

2. Experimental and results

2.1. Cloning

Cloning of each of the *N. meningitidis dsbA* genes was achieved using the ROBIOMOL platform (Institut de Biologie Structurale, Grenoble, France).

PCR amplification was achieved with Phusion enzyme (Finnzymes) using a template containing the different recombinant neisserial *dsbA* genes (*nmb0278*, *nmb0294* and *nmb0407*) and specific primers containing the sequence essential for ligation-independent cloning. The PCR products were inserted into the vector pLIM01 (His_6 fusion, AmpR) by annealing to promote the cytoplasmic expression of the protein of interest in fusion with an N-terminal MAHHHHHHG-HHHQLENLYFQG tag (pLIM01) containing a tobacco etch virus (TEV) protease cleavage site. The ligation products were transformed into *E. coli* and the presence of the gene of interest in the vector was verified by restriction-endonuclease analysis. The nucleotide sequences encoding the periplasmic secretion signal and the membrane-anchor sequence of the DsbA1 and DsbA2 proteins were excluded (Fig. 1).

2.2. Purification

The three recombinant proteins were expressed and purified at 277 K according to the same protocol, except that all the buffers used to purify DsbA3 were supplemented with 500 m*M* NaCl in order to avoid protein aggregation.

Recombinant plasmids were transformed into *E. coli* BL21(DE3)-Codon Plus-RIL. 1 l LB broth medium containing 1 m*M* ampicillin was inoculated with 20 ml of an overnight culture at 310 K. At an OD_{600} of approximately 0.6, expression of the recombinant enzymes was induced by the addition of 1 m*M* IPTG and cells were grown for an additional 3 h. Cells were pelleted by centrifugation (5000*g*), resuspended in 10 m*M* HEPES pH 7.0 (buffer *A*) and disrupted using a French press. The soluble fraction was recovered by centrifugation at 18 000*g* and mixed with 5 ml of a suspension of Ni–NTA resin (Qiagen) pre-equilibrated with buffer *A* at 277 K. The resin was then loaded onto a column. To remove unbound proteins, the resin was extensively washed with 50 ml buffer *A* containing 50 m*M* imidazole. The proteins were eluted using a linear gradient of buffer *A* containing 50–400 m*M* imidazole. The pooled peak fractions were dialysed against buffer *A* and incubated in TEV buffer (50 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA, 1 m*M* DTT) with recombinant His_6-tagged TEV protease in a 1:100 molar ratio for 1 h at room temperature followed by 2 h at 277 K. The protein solution was dialysed against buffer *A* and loaded onto Ni–NTA beads to remove all His-tagged proteins. The purified proteins were then concentrated to approximately 10 mg ml^{-1} using Centricon devices (Amicon,

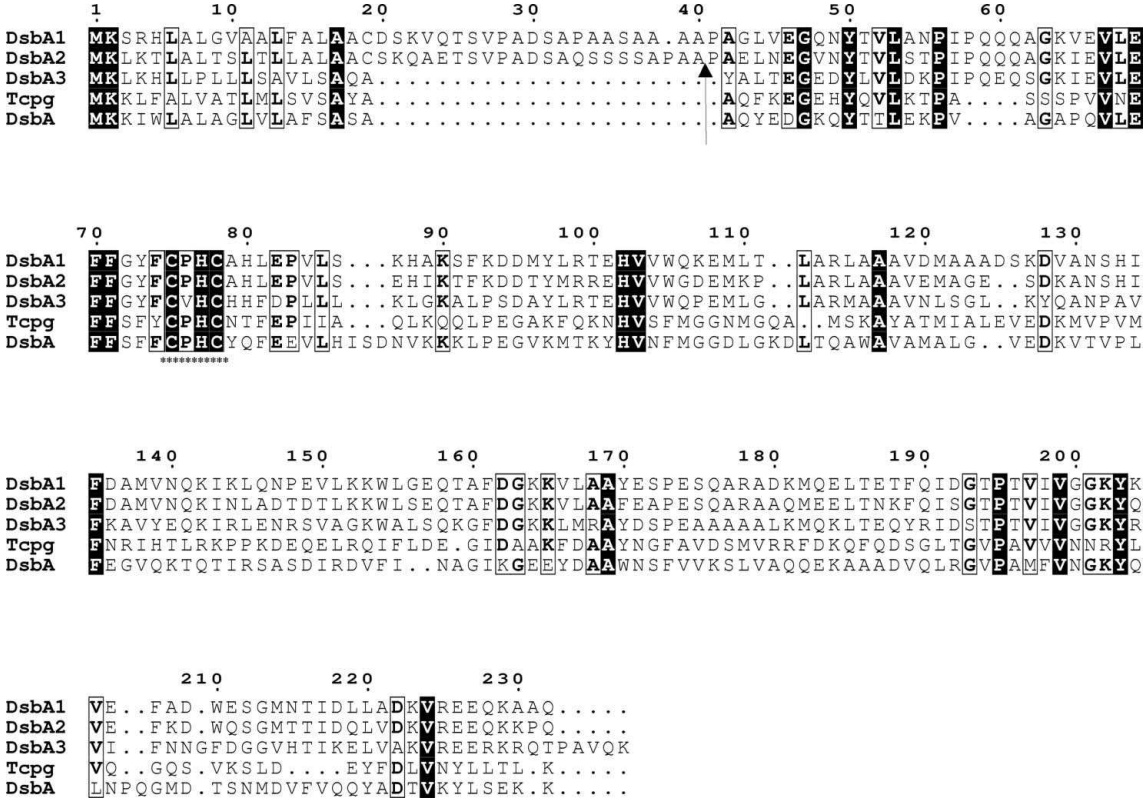

Figure 1
Sequence alignment of the DsbA homologues in *N. meningitidis* (DsbA1, DsbA2 and DsbA3), *E. coli* (DsbA) and *V. cholerae* (Tcpg). Identities (black shading) and highly conserved residues are highlighted. The arrow indicates the N-terminal residue of the three neisserial enzymes studied in this work. The conserved active-site tetrapeptide, Cys-*X*-*X*-Cys, is underlined.

10 000 Da cutoff) and loaded onto a size-exclusion chromatography column (Hiload Superdex 75, 16/60, GE Healthcare) for the final step of the purification procedure. The column was equilibrated with buffer *A* and the pooled peak fractions were concentrated to 12 mg ml^{-1}. The purity of the protein solutions was confirmed by SDS–PAGE (Fig. 2). The molecular weights obtained by mass spectrometry were 21 477, 21 461 and 21 836 Da for DsbA1, DsbA2 and DsbA3, respectively. The final protein concentrations were determined by UV spectrometry assuming molar absorption coefficients at 280 nm of 24 075, 22 585 and 24 535 M^{-1} cm^{-1} for DsbA1, DsbA2 and DsbA3, respectively. The final yield of each enzyme was 50 mg pure protein per litre of LB medium.

2.3. Oxidoreductase activity of the recombinant enzymes

To check the oxidoreductase activity of the three purified recombinant proteins, we tested their ability to catalyze the reduction of insulin interchain disulfide bonds by dithiothreitol (Holmgren, 1979). This reduction leads to insulin B-chain precipitation, which was monitored by the change in absorbance at 650 nm. Bovine insulin was concentrated to 1.7 m*M* in 25 m*M* HEPES and the final pH of the solution was adjusted to 8.0. A typical reaction mixture (0.2 ml) contained 150 µ*M* insulin in 100 m*M* potassium phosphate pH 7.0, 2 m*M* EDTA and 1 m*M* DTT. The precipitation of insulin was measured after the addition of 5 µ*M* of each enzyme and compared with a control that did not contain any protein. Fig. 3 shows that for all the DsbAs the reaction lag time is shorter than with the control, indicating that the three purified proteins exhibit thiol-disulfide oxidoreductase activity.

2.4. Crystallization

For crystallization, each protein was concentrated to 12 mg ml^{-1} in buffer *A*. As in the purification steps, buffer *A* was supplemented with 500 m*M* NaCl for the crystallization of DsbA3. Initial trials were performed using the sitting-drop method and commercial crystallization screening kits from Qiagen and Hampton Research. For the initial trials, we used a Tecan Genesis robot coupled with 96-well plates. 1 µl protein solution was mixed with 1 µl reservoir solution and the resulting drops were equilibrated against 100 µl reservoir solution. After optimization of the conditions in larger crystallization boxes (NeXTaL, 1 ml reservoir), the best DsbA1 crystals grew at room temperature in 2.4 *M* ammonium sulfate, 0.1 *M* bicine pH 9.0 or in 1.6 *M* trisodium citrate as thick plates or tetragonal bipyramids (Fig. 4*a*). DsbA2 crystals appeared in 2.4 *M* ammonium sulfate, 0.1 *M* MES pH 5.0 as hexagonal bipyramids at room temperature (Fig. 4*b*). DsbA3 crystallized as small rectangular prisms in 2.2 *M* ammonium sulfate, 0.25 *M* sodium malonate, 0.1 *M* MES pH 5.0 at 281 K (Fig. 4*c*). These crystals were routinely reproduced using the hanging-drop method of crystallization, mixing 2 µl protein solution with 2 µl reservoir solution.

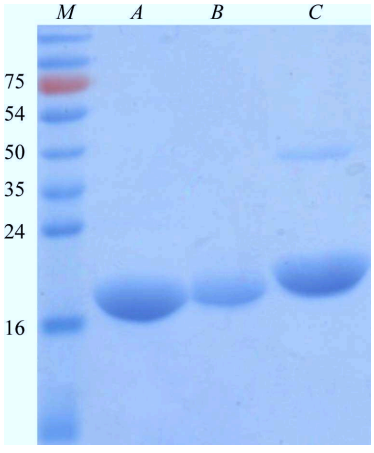

Figure 2
Analysis of the purified protein samples after gel-filtration chromatography. The purified samples were loaded onto a Coomassie Blue-stained 12% SDS–PAGE gel. Lanes *M*, *A*, *B* and *C* contain molecular-weight markers (kDa), recombinant DsbA2, DsbA1 and DsbA3, respectively.

2.5. Crystal parameters and X-ray diffraction

Prior to diffraction, the crystals were soaked in their respective crystallization solutions containing 30% glycerol as cryoprotectant. The crystals were then flash-cooled directly in liquid nitrogen. X-ray diffraction experiments were performed at the European Synchrotron Radiation Facility (Grenoble, France). Data were collected at 100 K on BM30 for DsbA1 crystals and on ID14-EH3 for DsbA2 and DsbA3. Diffraction data sets were processed using *MOSFLM* (Leslie, 1999) and intensities were scaled and reduced with *SCALA* (Evans, 1993) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Of the three proteins, only the DsbA2 crystals exhibited a poor diffraction pattern that did not allow us to determine the crystal parameters. The crystal parameters of DsbA1 and DsbA3 and their corresponding data-collection statistics are summarized in Table 1.

3. Conclusion

In this work, we cloned and purified the three DsbA enzymes from the pathogenic bacterium *N. meningitidis*. All the purified proteins were expressed at high levels in soluble form, lacking N-terminal signal sequences, in the cytoplasm of *E. coli*. The insulin-precipitation assay commonly used to test DsbA activity (Holmgren, 1979) shows that the three neisserial enzymes exhibit similar oxidoreductase activity and that the removal of the membrane anchor of the two lipoproteins does not abrogate this. The neisserial DsbAs are highly

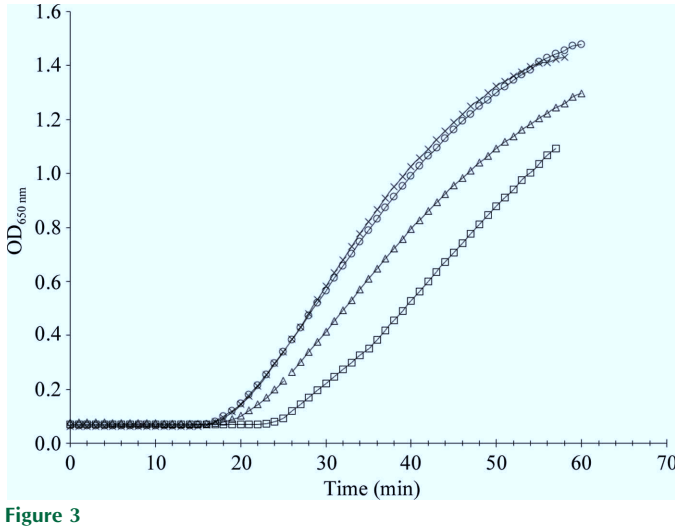

Figure 3
Assay of the disulfide oxidoreductase activity of meningococcal DsbAs. The precipitation of insulin was measured after the addition of 5 µ*M* DsbA1 (white triangles), DsbA2 (white circles) or DsbA3 (crosses). The absorbance owing to background precipitation of insulin by non-enzymatic reduction was measured in control tubes containing DTT but no added protein catalyst (white squares). The lag times measured for the control and the three meningococcal DsbAs are around 22 and 17 min, respectively.

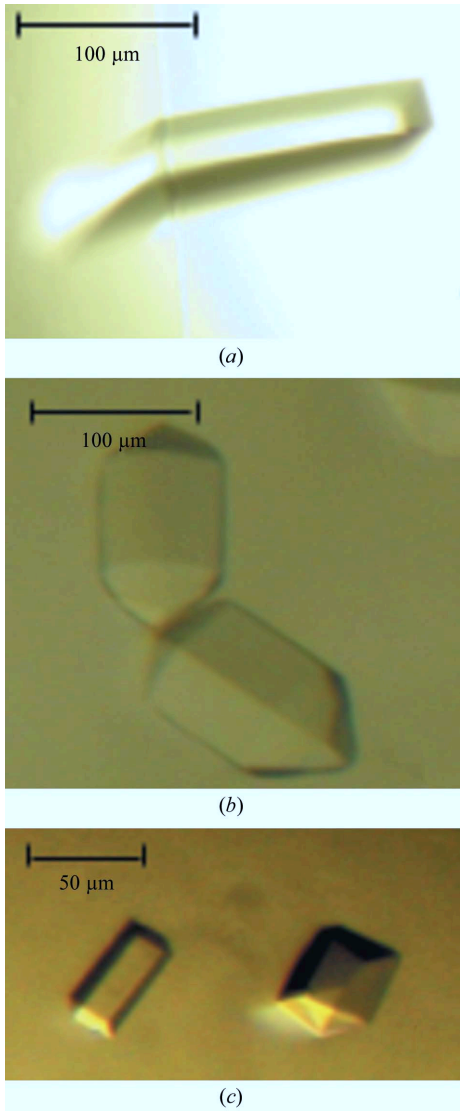

Figure 4
Crystals of the three *N. meningitidis* DsbA enzymes. All the enzymes crystallized in the presence of ammonium sulfate. Detailed crystallization conditions are described in the text. (*a*) DsbA1, (*b*) DsbA2, (*c*) DsbA3.

homologous; in particular, the two anchored membrane proteins are more than 70% identical. Accordingly, all three proteins crystallized under similar conditions requiring a high concentration of ammonium sulfate. This requirement is markedly different from the conditions employed for the successful crystallization of the *Vibrio cholerae* or *E. coli* DsbAs in polyethylene glycol (Martin, Waksman *et al.*, 1993; Hu *et al.*, 1997). The neisserial enzymes share 21% sequence identity with their *E. coli* and *V. cholerae* counterparts, the crystal structures of which are known (Martin, Bardwell *et al.*, 1993; Hu *et al.*, 1997). While this identity level might appear sufficient to solve the neisserial enzyme structures by molecular replacement either with the *E. coli* or *V. cholerae* models, no obvious solution has been obtained so far for DsbA1 or DsbA3 using this method of phasing.

Table 1
Crystal parameters and data-collection statistics calculated using *SCALA* (Evans, 1993).

Values in parentheses are for the last resolution shell.

Protein	DsbA1	DsbA2	DsbA3
N. meningitidis B serogroup gene	*nmb0278*	*nmb0294*	*nmb0407*
Space group	$P2_12_12_1$	Not determined	$I4_122$
Unit-cell parameters (Å)	a = 44.08, b = 46.42, c = 79.93		$a = b$ = 160.0, c = 76.80
Molecules per ASU†	1		2
V_M (Å^3 Da^{-1})	1.9		2.8
Solvent content (%)	35		56
Resolution (Å)	20.5–1.5 (1.58–1.5)		20–2.8 (2.95–2.8)
ESRF beamline	BM30A		ID14-EH3
Wavelength (Å)	0.979		0.931
R_{merge}‡	0.064 (0.241)		0.102 (0.282)
$\langle I/\sigma(I)\rangle$	6.8 (3.1)		6.1(2.7)
Total observations	274425		162296
Unique reflections	26907		12695
Multiplicity	10.2		12.9
Data completeness (%)	99.5		100

† Estimated from the Matthews coefficient value (Matthews, 1968). ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of the intensity of the hkl reflection and $\overline{I(hkl)}$ is the average intensity obtained with these i measurements.

While it is clear on the basis of the sequence that the three neisserial proteins exhibit the DsbA general fold, it seems highly likely that their three-dimensional structures include some significant differences from the *E. coli* and *V. cholerae* enzyme structures. Heavy-atom derivative research is currently in progress in order to solve these crystal structures.

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